MICROBIOLOGY

Project title: Survey of Naegleria

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Objective: Identification of thermophilic amoebae is important because of their possible impacts on human health. Recently, techniques have been developed to identify potential pathogens using modern molecular biological methods. These highly specific methods allow detection of amoebae from DNA isolated from multiple environmental samples, saving time compared to traditional isolation and culture techniques in which organisms are identified on the basis of morphology and physiology. Thus, molecular techniques allow a much more rapid and comprehensive survey of amoebae that may be present in thermal pools and are much less labor intensive. Furthermore, only small, easily collected water samples are required for processing. We propose to survey areas in YNP where people are soaking in thermal waters (either legally or illegally) for potential amoebic pathogens that pose substantial risks to humans. Information obtained from this study will fit well with the NPS goal of increasing scientific research within NPS ecosystems, inventorying species, and monitoring Park conditions. Furthermore, data obtained from this study will allow Park officials to make informed decisions regarding public health in the Park.

Findings: We began surveying areas in the park in late summer and fall of 2001. We sampled at Nymph Creek, the Boiling River, the swimming area along the Firehole River, the Madison River at the Madison Campground, and Kelly Warm Springs (in GTNP). Preparation of DNAs for PCR amplification is complete and we have begun to amplify the DNAs with taxonomically informative primer sets and to create clone libraries. We have positive PCR reactions for Nymph Creek and the Madison Campground so far, but we have not completed the analysis on all samples. We hope to complete the sequence analysis on the positive samples in the near future.

Project title: Isolation and Characterization of Thermophilic Microorganisms

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Objective: To determine the presence and ecological significance of thermophilic microorganisms of the greater Yellowstone Ecosystem. To obtain additional thermophilic microorganisms for microbial and biochemical studies including the purification and characterization of thermostable enzymes.

Findings: The field work in Yellowstone National Park involved a limited collection (during the last week in June) of microbial mat and water samples from previously sampled areas in White Creek and other areas as part of a long term thermophilic microbial populations study.

During 2001 collections were also made from Western Colorado Hot Springs where apparently new isolates of Exiguobacterium bacteria had been obtained (e.g. Pinkerton Springs seven miles north of Durango, Colorado). 16S rRNA sequence of these isolates have been deposited in the Gene Bank (Accession # AY047481) and a culture deposited in the American Type Culture Collection as *Exiguobacterium aurantiacum* var. Colo. Road (BAA-333). Reported at the 2001 national meeting of the American Society for Microbiology—Abstract I-92—Isolation and Characterization of a Gram Positive, Non Spore-forming Exiguobacterium-Like Organism Isolated from a Western Colorado Hot Spring.

Similar type of isolates have also been recently obtained from Southern India Hot Springs (Ganeshpuri, northeast of Bombay [Mumbai] India) and it appears that Exiguobacterium type isolates are also present in lower temperature Yellowstone hot springs (e.g. Huckleberry Hot Springs south of the South Entrance to Yellowstone National Park). However, for the present, the study of these and other Yellowstone microbial isolates is inhibited by not being able to deposit any proposed new isolates in the American Type Culture Collection (for study by other investigators etc.) because of a proprietary dispute between the U.S. Department of Interior and the American Type Culture Collection regarding the legal standing of such isolates of "Yellowstone Microorganisms."

Project title: Analysis of a Eukaryotic Microbial Mat Community Across Environmental Gradients in a Thermal, Acidic Stream

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Objective: Two Nymph Creek sites, defined in terms of differing light, pH, and temperature will be extensively monitored over diurnal and seasonal time periods. Contemporary analyses, including modern microscopic methods and rRNA sequencing will be used to document microbial diversity and analyze the mat's microstructure at both sites. Changes in macro- and micro-scale environmental conditions in the bulk water and through the verticle aspect of the mats will be recorded.

Findings: 1) We used culture-independent cloning and sequencing of PCR-amplified 18S rRNA gene segments to survey algal populations in situ. We also isolated and characterized axenic *Cyanidiophyceae* and identified them by sequencing a portion of their 18S rRNA genes. The results suggest that a strict autotrophic *C. caldarium*-like algal population is prevalant in situ. We have also determined that the algal population changes along the thermal, pH, and sunlight gradients of Nymph Creek; while the predominant alga upstream is *Cyanidium*, *Chlorella* dominates the mat at the downstream site. 2) Our microscopic examination of samples from Nymph Creek initially showed vahlkampfiids and heterolobose amoebae. This observation prompted sequence analysis of cloned rRNA genes PCR-amplified directly from Nymph Creek and revealed the presence of vahlkampfiid species. A phylogenetic comparison showed a sequence that forms an independent lineage within *N.fowleri*-like protozoa that may represent a new, potentially pathogenic species. 3) We detected sulfite and sulfate reduction by anaerobic bacteria in Nymph Creek using molecular analysis and traditional culture-based methods.

Project title: Biomolecular Diversity in Yellowstone National Park

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Additional investigator: Martin Keller, Jay M. Short

Objective: Explore the microbial diversity at Yellowstone with special focus on the thermophiles that exist in Yellowstone's thermal features. Also of particular interest are microorganisms that exist

in thermal features that contain extremely low and high pH levels. The ultimate objective of our research is to discover new commercial products.

Findings: Unfortunately, we did not engage in any research activities in Yellowstone because we are still waiting for the completion of the Environmental Impact Statement.

Project title: Microbial Physiology and Ecology: DNA Damage and Photosynthesis

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Objective: The objective of this project is to study diurnal patterns of organismal physiology (e.g., photosynthesis, DNA synthesis) in order to better understand evolution on early earth and the way organisms function in their environment today. Specifically, in 1999 the focus was on the effect of two naturally occurring DNA damaging agents on DNA synthesis rates, ultraviolet radiation and hydrogen peroxide.

Findings: In 2000 research focused on the effects of UV radiation and hydrogen peroxide on microbial mat communities in Norris Geyser Basin and Octopus Spring, we found that UV radiation enhances DNA synthesis rates during the day, which we interpret as being indicative of excision repair. However, previous work suggests that the damage may be due to UVA effects mediated through oxidative damage rather than the direct effect of UVB. Experiments adding hydrogen peroxide to sample showed an increase in DNA synthesis in response to small amounts of additional hydrogen peroxide, and a decrease in response to high levels, with another increase at even higher levels, about 1 mM for Octopus and Zygogonium mat. At the very highest concentrations of H2O2, DNA synthesis, of course, drops to zero, probably an indication of cell death. For all the mats studied, DNA synthesis stopped by 1 M H2O2. Pre-challenging Zygogonium with H2O2 prior to measuring the effect of H2O2 on DNA synthesis decreased the subsequent rate of DNA synthesis. This is suggestive of an induction of catalase. Techniques for studying levels of catalase and superoxide dismutase were begun in collaboration with Vanessa Lancaster and Bob Blankenship, Arizona State University. These studies will be repeated and extended in 2002.

The effects of several drugs were tested on the effect of H2O2 on DNA synthesis. Caffeine (1 mM) increased DNA synthesis in the presence and absence of additional H2O2 in Cyanidium, Zygogonium. Zygogonium mats that were placed under UV opaque screens from September to June 26, 2000 showed a down regulation in DNA synthesis when finally exposed to solar radiation in contrast to mat that was left under the UV opaque screen.

Project title: Isolation of New Hyperthermophiles and Investigations of

Hyperthermophilic Biotopes

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Objective: Isolation of new hyperthermophiles and investigations of hyperthermophilic biotopes.

Findings: From hot acidic soil samples, taken in 2000 in YNP, acidophilic members of the Aquificales order were enriched and isolated. This result demonstrates for the first time the existence of acidophilic Hydrogenobacter-relatives in YNP. Two publications on this topic are submitted.

During a field trip in YNP, samples from Obsidian Pool were collected for microbiological investigations.

Project title: Molecular Assessment of Microbial Communities in Hot Spring Structures and Their Responses to Light Manipulation

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Objective: To determine microbial community structure in lithifying structures within hot springs runoffs.

Findings: Denaturing Gradient Gel Electrophoresis (DGGE) and subsequent sequencing of partial 16S rRNA gene sequences was used to investigate the molecular biodiversity of cyanobacterial communities inhabiting various lithified morpho-structures in a hot spring of Yellowstone National Park. These morpho-structures flat-topped columns, columnar cones, and ridged cones resemble ancient stromatolites *(Conophyton)*, which are presumed to be biogenic. The top, middle and bottom sections of these lithified morpho-structures, were analyzed to determine the vertical and spatial distribution of cyanobacterial communities. Results from DGGE indicate that the cyanobacterial community composition of lithified morpho-structures (flat-topped columns, columnar cones, and ridged cones) were largely similar in vertical distribution as well as among the morpho-struc-

tures studied. Analysis of partial 16S rRNA gene sequences obtained from these community profiles show that the closest relatives of these lithifying cyanobacteria are detected or isolated from hot springs. Preliminary results also indicate that the cyanobacterial communities in these lithified morpho-structures were significantly different from communtities in surrounding non-lithified mats. These results provide additional support to the theory that certain Phormidium/Leptolyngbya species are involved in the morphogenesis of lithifying morpho-structures in hot springs and may have played a role in the formation of extant lithified stromatolites as well as ancient ones.

Project title: Development of Harsh Environment Biosensors

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Objective: The major objective for this project was to develop more robust biological sensors for use in harsh environments. These environments might include high temperatures, high or low pH, or high salt conditions where conventional biological sensors fail rapidly due to denaturation of the biological component. Our approach was to isolate and purify enzymes from thermophilic microorganisms and utilize these enzymes for sensor development. These enzymes have evolved to be stable at high temperatures and would yield a biological sensor that could operate at elevated temperatures for extended periods of time.

Findings: We have been working to purify a thermostable catalase from an organism (*Thermus brockianus)* isolated from Yellowstone hot springs previously. We have partially purified this enzyme with ion exchange, hydrophobic interaction, and gel filtration chromatographies. Our initial characterization studies have shown the enzyme to have optimum activity at a temperature of 80°C and a pH of 8.0. The enzyme is active from 30°C up to at least 100°C and in the pH range from 6.0 to 10.0. The enzyme is also stable for at least seven days when incubated at 70°C. We plan to continue characterization of this enzyme including determination of the molecular weight, enzyme kinetics, and inhibitors. Future work will focus on the development of a biosensor using this enzyme.

Project title: Isolation, Identification, and Characterization of Microorganisms Living in Extreme Environments

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Objective: There are two main project objectives. First, to train students in the safe and proper sampling of hot spring environments for thermophilic microbes, as well as characterizing dissolved components in the spring waters for media development. Secondly, this study involves laboratory characterization of culturable microorganisms from the hot spring samples as well as looking at species diversity using denaturing gradient gel electrophoresis.

Findings: Sample collection and removal from the Park was not permitted under the conditions of my permit as stipulated by Ann Deutch. However, I was allowed to take students to Snake Hot Springs, Shoshone Geyser Basin, and Spray.

Samples were collected from the Bear Creek thermal area outside the Northern boundaries of the Park and characterization of the microbial inhabitants is ongoing. These springs although classified as thermal springs only have temperatures ranging from around 30–35°C degrees celsius with pH values between six and seven.

An inventory of organisms that we have identified from samples taken in previous years is being prepared for submission to Yellowstone National Park for the Thermophile Inventory database.

The status of these studies is ongoing.

Project title: Spectral Analysis of Hyperthermophile Organisms

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Objective: To define spectral reflectance character of hyperthermophile mat communities; determine if each community has a diagnostic spectra; determine spectral signature of the sinter; and determine if a biologic signature occurs in the sinter which persists with time. Because each organism has specific chlorophyll, bacteriochlorophyll and carotenoid compositions, each has a well defined spectral signature. Data are of sufficient spectral resolution to allow pigment identification.

A long term goal is to use spectral signatures as a reconnaissance tool to study community structure at springs that have not been examined. If, indeed, each community has a diagnostic spectra, then such data can be quickly collected at unstudied springs allowing an assessment of the organisms that are present. While this technique does not identify all organisms, it does allow a rapid evaluation of general population and any significant presence of organisms that have not been previously recognized.

Spectral data are collected field spectrometer in the range 350–2,500 nm. Mat reflectance data are collected in situ. In a few cases, small amounts of mat are removed, measured and replaced.

Typically, a fiber optic is mated to an artificial light source and placed just above the mat. This results in a field of view of a few cm. An artificial light source is used to reduce the effect of water vapor on the spectra. Because of changing cloud conditions and significant water vapor present at the springs, the spectra of natural sunlight will rapidly vary. Water vapor produces deep absorptions at several wavelength making it difficult to correct the observations. An artificial light source reduces the path length of light and therefore the influence of water vapor.

Data are collected from the source to a point where the water cools to ambient temperature. Stations are located at regular intervals on the channel or at obvious changes in the mat. This ensures that changes in the community structure are observed. At each station, the color and morphology of the mat surface are noted along with temperature and pH. Several spectra are collected at each spot and several spots are measured at each station. This avoids the problem of bad data and ensures that local variation in the spectra will be defined. In a few locations, the surface mat is scraped away allowing data to be collected for the subsurface communities. Disturbed areas which reveal underlying mat material are also examined.

In addition to observations of the mat communities, data are also collected on the silica and carbonate sinter that forms around the margin of the spring, that line the outflow channel and older materials in the immediate areas. The purpose of these measurements is to define the spectral character of the sinter and to determine the character of any biologic material that might be present but not visible. Dried, inactive sinter is also examined to determine if any organic material can be detected.

A new objective of the investigation will be to use an ultraviolet spectrometer to examine the margins and interiors of certain springs. SEM images of the sinter have revealed the presence of biofilms and microfossils, yet the temperature of the water at these locations is above that which would be expected to support life and in areas where there is no obvious mat. An ultraviolet fluorescence spectrometer that can withstand submersion into very high temperature water will be used to determine if organic material is present around the rim of the spring or lining the walls of the pool. The spectrometer illuminates the surface with an ultraviolet light source. Organics will fluoresce at specific wavelengths which can be detected by the instrument. This will allow a determination of whether organic materials are present in large quantities, which types of materials are present, and if they occur both in the pool and on the rim or only on the rim.

Findings: To date we have collected spectral data at a variety of springs spanning a large temperature and chemistry range. Sites examined include: Octopus Spring, Mushroom Spring, and various springs up White Creek above Octopus; along Nymph Creek between the source and the Nymph Lake; at several springs and channels in the Lemonade Creek area, in a number of locations in the Norris Geyser Basin both adjacent to the boardwalk and in the area around Cinder Pool; at the Chocolate Pots mounds, and several springs in the Mammoth area. In each case a series of stations were set up beginning at the source and extending down the channel to a point where the water temperature reaches ambient, where the mat disappears, or where the flow enters another flow. At each station several measurements are made to examine the extent of local variation in the spectral character.

At Octopus Spring, the *Synechococcus*, *Chloroflexus* and *Phormidium* communities each have a well defined spectral signature. The mat has been examined during two years and the results from the two sets of data are consistent. *Synechococcus* exhibits well defined absorptions at 625, 675, 740, 800 and 880 nm. *Chloroflexus* absorptions occurs at 597, 683, 747, 798 and 890 nm. *Phormidium*

has absorptions at 675, 740, 798 and 874 n.

At a test of the ability to determine whether the spectra could be used in a reconnaissance mode, two springs (Tuft Geyser and an adjacent unnamed spring) upstream from Octopus, along White Creek, were examined. Tuft Geyser has a narrow outflow channel with a green and orange mat; an adjacent pool has a short effluent channel with a surficial green mat and an underlying red-orange mat. Spectral data indicate that the mat associated with Tuft Geyser is composed of *Synechococcus* at the high temperature end and at *Phormidium* at lower temperatures. The mat along the channel from the adjacent spring is interpreted to be a *Synechococcus/Chloroflexus* mat community similar to that at Octopus Spring.

The outflow at Nymph Creek between the springs and the lake has been examined. This spring has a very low pH (2.9). Immediately surrounding the sources there is a yellow deposit and local filaments. The yellow material is sulfur. A short distance downstream a green mat composed of *Cyanidium caldarium* occurs. Spectra of this mat indicates that while there are morphologic and visible color variations, the spectra remain largely the same. At the lower end of the stream, the *Cyanidium* is replaced by *Chlorella* which has a significantly different spectral character. Lemonade Creek has a similar acidic water supply and the same characteristics are observed there; yellow deposits around the sources are considered to be sulfur and the green mat is interpreted to be *Cyanidium*.

To test the ability to predict the biota in other acidic springs, data were collected at several spring and outflow channels in the Norris Basin in the area of Cinder Pool. Here, numerous springs occur with yellow deposits around the source and thin green mat in the effluent. Spectral data indicate these are sulfur and *Cyanidium*.

At the Chocolate Pots, the data indicate the presence of biota across the entire mound surface, even where there is no obvious mat. An absorption near 680 nm, indicative of chlorophyll is observed. The mineral material that forms the dark brown mound is ferrihydrite with the possible addition of some other iron bearing phases.

Data were collected from a number of springs and seeps in the Mammoth area to examine the biota in an environment where carbonate is mineral deposited rather than silica. These springs also contain a green version of *Chloroflexus* rather than the red version typical of Octopus Spring. These data have been collected but not interpreted.

Project title: Diversity and Habitat Range of Sulfate-Reducing Microorganisms

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Objective: Our research at Yellowstone National Park has focused on better defining the diversity of sulfate-reducing bacteria along environmental gradients of pH and temperature. Organisms having the capacity to respire sulfate drive a key step in the global cycling of sulfur and are likely an important biological presence in many of the sulfur-rich geothermal areas within Yellowstone National Park. A long-term objective is to better define the environmental limits of dissimilatory sulfate reduction. Our primary method of assessing population diversity has been comparative sequence analysis of the highly conserved dissimilatory sulfite reductase (DSR) gene. This gene can be selectively amplified from DNA recovered from site material using PCR, as reported by our research group (Wagner, Roger et al. 1998; Minz, Flax et al. 1999). Comparative sequencing of cloned DSR genes avoids the usual biases associated with culture-based methods of characterization. We complement this molecular characterization with on-site activity measurements and also use more traditional culture-based methods to evaluate cultivable sulfate-reducing bacteria.

Findings: The discovery of deeply diverging phylogenetic lineages of sulfate-reducing bacteria, as inferred from DSR gene sequence divergence, suggests that our current understanding of this important functional group of microorganisms is incomplete. Our combined analyses of different regions throughout the Park indicate that sulfate respiration is a significant biogeochemical process in many of Yellowstone's geothermal features. In 2001 we followed up on studies performed during the previous three years of the study in two established field sites and in a new study set of hot springs. In Mushroom spring we repeated experiments investigating the activity and diversity of sulfate-reducing bacteria found in the microbial mats present in the effluent channel. As observed in 2000, although these mats are relatively low in sulfate, they sustain very high rates of sulfate reduction. Molecular analyses, using PCR to selective recover bacterial 16S rRNA sequences from the Mushroom Spring mats revealed high bacterial diversity. Coincident analyses of DNA sequences encoding for the dissimilatory sulfite reductase confirmed significant diversity of sulfate-reducing bacteria in this spring as well. We also followed up on previous activity assessments at the spring we refer to as Black Sediment pool in the vicinity of Nymph Creek. Significant endogenous sulfate reduction rates were once again observed in this spring. Finally, we explored a few new sites in the vicinity of Crater Mountain and tested them for endogenous sulfate reduction activity (results pending).

Project title: Molecular Ecology of Photosynthetic Hot Spring Bacteria That Resemble *Heliothrix oregonensis*

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Additional investigators: Daniel Lodge

Objective: 1) To perform an ecological survey of thermal features in Yellowstone in order to describe the distribution of Heliothrix-like organisms. 2) To isolate DNA from biological samples that contain Heliothrix-like organisms. 3) To utilize PCR to isolate 16S rRNA genes from these DNA samples. 4) To clone, characterize, and compare representative 16S rRNA genes at the molecular level.

Findings: This year, we reported and published extensive molecular results from five long-term study sites (Hillside, Spray, Fairy, Witch, Western Pool). We also reported and published our implementation of this research project in our undergraduate teaching curriculum for molecular and microbiology coursework. In terms of new studies, we have three, all of which are in progress based on materials collected on this annual permit: 1) To address potential origins for red layer communities, we collected water samples from four long-term study sites and are in the process of analyzing 16S rRNA clones from sourcewater. 2) To visualize and demonstrate co-localization of retrieved DNA sequences with bacteria in the mat, we collected and fixed mat specimens for current in situ labeling studies, all of which are in progress. 3) To expand our ecological survey for new red layer communities, we collected new mat samples from two sites at Joseph's Coat thermal basin during a comprehensive GIS-based survey of this fascinating region. These samples have yielded informative bacterial sequences and we anticipate reporting and publishing this data alongside general GIS survey information in the upcoming year. All information has been or will be archived on our "Red Layer Microbial Observatory Database."

Project title: Analysis of Metal Resistance in Yellowstone Bacteria

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Additional investigator: David Barrie Johnson, Mark Delwiche, Heather Silverman, Deborah Newby, Amber Miller

Objective: Isolation and characterization of thermophilic, thermoacidophilic, and other bacteria

and archaea from locations throughout Yellowstone to identify these microorganisms on the basis of 16S rDNA sequences and determine mechanisms of resistance/tolerance at the genetic level. Isolates may also be used in other INEEL research (V. Thompson, W. Apel, F. Roberto, co-PIs) screening for novel enzymes.

Findings: Three stable enrichment cultures have been obtained from 2001 sampling activities in and around Norris Geyser Basin and Crater Hills. Pools from which these enrichments (maintained at 700°C and an initial pH of 2.0) had measured temperatures of 850°C and pH 1.5-2. GPS coordinates and photodocumentation of these sites in August, 2001, are also available for these sampling locations. Initial characterization of microorganisms present based on 16S rDNA sequencing indicates these organisms to be members of the genus Acidianus. Collaborative work with other researchers is examining the distribution of archaeal viruses associated with these microorganisms (at least nine different morphologies have been observed to date). Studies of the microorganisms and associated viruses are ongoing.

Project title: Characterization of the Microbial Rhizosphere Population of Acid and Thermotolerant Grasses Associated with Hot Springs and Microbial Diversity in Thermal Soils in YNP

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Additional investigators: Lina Botero, Tracy Norris, Mark Burr, Jesse Donohoe-Christiansen

Objective: To study the diversity and identification of the thermophilic and acidophilic organisms associated with thermophilic plants located in YNP. We are also very interested in examining the diversity of the microbial community that thrives in select thermal soil locations.

Findings: We have obtained molecular evidence that some thermal soils (Temp. = 650°C to 850°C) apparently have diverse and complex prokaryotic communities. This study is continuing as we are developing new culturing techniques to cultivate maximal numbers of different prokaryotes from these soils.

Physiological and biochemical characterization of these different isolates is on going. Minimal soil disturbance has occurred; typically we use 1–5 gram of soil. We have discovered molecular evidence of extremely thermophilic *Pseudomonas putida* and *P. synxantha* (RNA-based RTPCR clones), and we have been able to cultivate these pseudomonads as part of a biofilm community. We have isolated a new extremely thermophilic bacterium having closest phylogenetic affiliation with a cluster of environmental PCR clones within the green non-sulfur division. Additional rRNA gene-based molecular work has uncovered RTPCR clones of Archaea that are only distant matches (79–81% sequence identity) to other Archaea sequences.

Project title: Microbial Biotransformations and Ecology

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Objective: To isolate microorganisms with unique metabolic activities allowing for the transformation of C-1 compounds, polycyclic aromatic compounds, and related products from the petroleum industry.

Findings: More than thirty strains of bacteria were isolated from thermal features in Yellowstone National Park that are capable of growing on C-1 compounds. We are currently conducting experiments with two isolates that are capable of growth at temperatures as high as 550°C, using methane as the sole carbon source. We believe these isolates possess the soluble form of the enzyme methane monooxygenase and could be useful in biocatalytic production of methanol at high temperatures. We are concluding experiments to further characterize these organisms based on internal membrane structure, 16S rDNA sequence, and functional gene analysis.

We have also characterized microbial structure in thermal features sampled during August 2000. DNA extracted from these samples was amplified by the polymerase chain reaction. We used denaturing gradient gel electrophoresis (DGGE) to separate DNA fragments based on nucleotide base sequence. In this way, we obtained a profile of genetic variability in these samples. We found that microbial diversity was greater in neutral to high pH thermal features than in acidic features, and there were changes in the composition of the microbial community related to pH. Surprisingly, diversity did not decrease at high temperatures, nor did the composition of the microbial community change as a function of temperature.

Project title: Effects of UV Radiation, Desiccation, and Heavy Metals on the Photosynthetic Microorganisms of Hot Springs and Associated Sediments

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Objective: The principal objectives that frame our proposed work are: A. Research concerned with Cyanidium and relatives. 1) Characterize *Cyanidium/Galdieria/Cyanidioschyzon* isolates to collect physiologic and phylogenetic criteria to unambiguously establish the genera and species that make up the Cyanidium complex and to distinguish them from other thermoacidophilic eukaryotic algae. 2) Determine if environmental features (e.g. heavy metals, pH, temperature, solar irradiance, competition, desiccation) may act as barriers to dispersion, or are correlated with specific *Cyanidium/ Galdieria/Cyanidioschyzon* ecotypes within the various thermoacidic habitats.

B. Research concerned with cyanobacterial crusts near hot springs: Assess the contribution of scytonemin to the survival of cyanobacteria under desiccated conditions.

Findings: A. The work with *Cyanidium* and related eukaryotic algae involved the collection of live specimens from numerous thermoacidic habitats over much of Yellowstone National Park. Isolations of many strains were accomplished, and the ribosomal 18S DNA was sequenced for some of these, and at this point the nearest relatives of a few strains are closest to two species of *Galdieria*. Preliminary studies have shown that heterotrophic growth of most of the strains is negligible; some are unable to grow with nitrate as the nitrogen source; and growth on soil and water taken from various native sources containing heavy metals was slow to nil, but differed among strains (work done at Montana State University and University of Oregon).

B. The work with cyanobacterial crusts is in a very preliminary stage, and most of the work involves experiments with culture isolates at the University of Oregon.

Project title: The Biogeochemistry of Sublacustrine Geothermal Vents in Yellowstone Lake, WY

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Additional investigators: Carmen Aguilar, Robert Paddock, Russell Cuhel, James Maki, Charles Wimpee, Patrick Anderson

Objective: Principal Hypotheses: 1) Yellowstone vent throat habitats are capable of sustaining thermophilic microbial communities in situ, resulting in gradients of thermophilic to extreme thermophilic bacteria with different nutritional characteristics within vent conduits, from deep in the conduit to the mouth of the vent. 2) Vents in different basins will support different types of extremo-

phile communities because each biogeochemical domain provides vent water sources of differing composition with respect to energy sources (e.g., reduced iron, manganese and sulfur; hydrogen; methane). 3) Significant microbial activity occurs in vent field sediments, resulting from seepage of vent fluids and fracture zones.

Findings: Mineral inputs to Yellowstone Lake, WY come from a variety of sources, including hydrothermal vents, ground water, rainwater, flux from sediments and direct runoff. One third of Yellowstone Lake is directly influenced by hydrothermal activity (hot water vents and fumaroles). Geothermally heated water percolating through the chamber is highly enriched in carbonate, silicate, chloride, and methane, with some locations additionally rich in iron and sulfide.

Microorganisms that live in high temperature ecosystems are tightly coupled to their environment. A detailed understanding of the geochemistry of hydrothermal environments can be an important component in deciphering critical characteristics for the presence of microbial life under these changing conditions.

More than 25 chemosynthesis incubations included more than 20 vent samples and an array of associated water column samples. Due to weather and scheduling constraints, the West Thumb and Mary Bay areas of the lake were intensely sampled while Stevenson Island and outlying areas near Mary Bay were postponed until 2002. This permitted us to apply more detailed analyses of chemosynthesis in the two regions than had been possible in previous years. Especially improved in 2001 (but not yet optimized) was examination of high temperature chemosynthesis (50–700°C) in parallel with in situ temperature incubations.

Vent waters in West Thumb typically contained sub-micromolar concentrations of Fe while those in Mary Bay and off Stevenson Island contain up to 10M. The water column concentrations of dissolved Fe range from 250–450 nM in Mary Bay, but were below detection (180 nM) in the waters of South East Arm, West Thumb, and off Stevenson Island.

Pore water and vent water chemistry provide evidence for lake water dilution of vents below the sediment-water interface. Significant fracturing of source water conduits was indicated by extreme differences in pore water profiles from cores less than five m apart in geothermally vigorous West Thumb. Some samples approached theoretical reservoir composition for conservative geochemical tracers.

Porewater results from the geothermally active areas of Mary Bay and West Thumb show Cl- enrichments reaching several mmolar and, in the case of Mary Bay, extrapolate to the geothermal end member (~ 20 mM) at a Departmenth of only 2–3 m. These steep concentration gradients support diffusive Cl- fluxes across the sediment-water interface three orders of magnitude higher than those in non-venting depositional areas.

Project title: A Survey of *Pilobolus* from Yellowstone National Park

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Objective: The objectives for the "A Survey of *Pilobolus* from Yellowstone National Park" are: 1) To determine what species of *Pilobolus* can be isolated in Yellowstone National Park. 2) To compare the various isolates for differing morphological characteristics of the isolates found. 3) To determine if the taxa from Yellowstone are the same as those found in different geographic areas. 4) To compare isolates of various taxa from various locations by contrasting morphological characters to DNA sequences and short chain fatty acid composition.

Findings: During 2001 isolates of *Pilobolus* were collected in Yellowstone National Park during May and August. These isolates were collected from mule deer, and elk from areas near Slough Creek, south of Solfatara, Roaring Mountain, the plateau north of Mammoth Hot Springs and about two miles south of Mammoth Hot Springs. All isolates maintained in the laboratory at Indiana University East are being used as part of larger studies to distinguish among the species of *Pilobolus*. Substantial progress was made in the past year using RFLP fingerprinting and DNA sequencing to resolve evolutionary relationships in *Pilobolus*. Two graduate student projects are in progress describing these techniques and the relationships found. It is hoped that these projects will be completed within the next couple of years. New fluorescent techniques have been attempted to show areas of growth in *Pilobolus* from Yellowstone. Results have been presented in Indiana Academy of Science meetings each of the past two years.

Project title: Bacterial Diversity of Thermophilic Anoxygenic Phototrophs

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Objective: The major objective of this research is to identify and culture photosynthetic microorganisms (anoxygenic photosynthetic bacteria) from Yellowstone thermal springs. In this regard, I am most interested in species of purple bacteria, green bacteria, and heliobacteria.

Findings: Several thermophilic photosynthetic bacteria have been isolated by my laboratory from Yellowstone thermal springs, most notably, the purple bacterium *Thermochromatium tepidum*, and the heliobacterium, *Heliobacterium modesticaldum*. All organisms isolated have been deposited in the American Type Culture Collection (ATCC, Manassas, VA), and are available for distribution to any qualified microbiologist. We continue enrichment and isolation experiments for new species, in particular, acidophilic photosynthetic bacteria.

No sampling was done in 2001 because we were scheduled to leave on September 12, 2001 for four days in Yellowstone and had to cancel due to the travel ban surrounding the terrorist attacks.

Project title: Geochemical Constraints on the Ecology of the Deep Lineages within the Bacteria and Archaea

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Objective: 1) To determine the microbial diversity and geochemistry associated with high temperature thermal springs in YNP. 2) Study the ecology of microbial communities inhabiting YNP thermal springs.

Findings: Our research in 2001 was focused on Calcite Springs. We collected geochemical and molecular biological samples along chemical and physical transects in the springs. Additionally, enrichment culture techniques were used to isolate novel thermophilic microorganisms, including the bacteria that form black filaments in many of the near-neutral springs in Yellowstone. Our research in 2001 will focus on linking geochemical and community differences and using our cultures to understand the physiological diversity of Calcite Springs.

Project title: Characterizing DNA Methylase and Restriction Enzyme Genes in Environmental DNA

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Additional investigators: Rick Morgan, David Ward

Objective: Restriction enzymes are one of the key reagents used in molecular biology. They recognize specific sequences within DNA and cleave at or close to this recognition sequence. More than 3,300 of these enzymes have been characterized worldwide, and more than 500 are available com-

mercially (Roberts and Macelis 2000). At present, we know of restriction enzymes that are able to recognize 240 different specific sequences. However, there continues to be an interest in finding both new specificities and new enzymes with specific properties that may make them especially suitable for certain applications.

We are interested in several outcomes from this research. First and foremost, we hope to isolate and characterize some new restriction endonucleases with novel properties, either ones that recognize completely new DNA sequences or far more stable isoschizomers of known enzymes that might be useful for specific applications where heat stability would be essential. Second, we hope to isolate and characterize DNA methylase genes, which comprise the second essential part of the restriction-modification systems we wish to study. We also aim to identify the organisms present in the samples studied by amplifying and sequencing the 16s rRNA genes present in the samples. To access the restriction-modification systems we isolate DNA from a thermophilic mat or filament sample, then prepare a DNA library from this DNA. We then employ one of several methods to identify clones that might carry restriction-modification genes.

Findings: One visit to Yellowstone was made in October of 2001, when, as the snow began to fly, samples of cyanobacterial mat, pool sediment and prokaryote filaments in run off channels were collected in the White Creek drainage area. The samples ranged from 500°C to 870°C and from pH 6.5 to pH 9. We are currently preparing DNA from the samples by a variety of methods, including bead-beating and chemical lysis. Construction of DNA libraries from the sample DNA is underway. The libraries will then be used for identifying restriction-modification systems. We have characterized a small number of 16s rRNA clones from Mushroom Spring, including several that are most similar (98% identity) to Synechococcus species isolated in Oregon by Miller and Castenholz (Appl Environ Microbiol 2000 Oct; 66[10]: 4222-9), several Chloroflexeae type sequences and Cytophagales sequences. Characterization of more 16s sequence clones is ongoing.

Project title: Protein Comparison of Thermophiles and Oral Bacteria

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Objective: Oral bacterial microflora are extremely diverse (more than 300 different species in the normal oral cavity) and have to survive relatively large temperature and nutritional variations. Thermophilic microorganisms have been fairly well described, but no comparison has been reported with oral bacteria. It is proposed here to compare protein antigens between thermophilic and oral bacteria by immunological and electrophoretic (protein size) techniques. SDS-PAGE electrophoresis will be used to compare the sizes of proteins between representative thermophiles and laboratory strains of oral streptococci (primarily *Streptococcus mutans*, the causative agent of human dental caries). Immunological assays such as ELISA and western blots will be used to compare reactivity between antibodies to protein antigens on *S. mutans* and the thermophiles. It is anticipated that similar proteins will be observed between thermophiles and oral bacteria implying a possible

common ancestry.

Findings: Bacterial colonies were isolated on both selective and non-selective petri plates. Selected colonies were propagated and stored frozen until assayed. Samples were collected from human volunteers to compare to Park samples. Preliminary analysis of SDS-PAGE gel results indicates few proteins of similar size between the oral *Streptococcus mutans* and the Yellowstone microorganisms.

Project title: Ecology of Hot Spring Microbial Communities

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Objective: We have continued our long-term efforts to study the diversity, ecology, evolution and physiology of microorganisms inhabiting hot spring microbial mats. In essence, we treat the microbial mats of alkaline siliceous hot springs as models in which to investigate fundamental questions about the composition, structure and function of microbial communities. We also compare mats found in these and sulfidic hot springs, which are modern analogs of stromatolites, the most abundant fossils of the Precambrian Era, in an effort to study the present to learn about the past. Our specific objectives are as follows: 1) To evaluate the hypothesis that adaptive radiation of ecologically distinct populations explains the diversity of phototrophic microorganisms detected in mats of alkaline siliceous springs (e.g., Octopus Spring, Mushroom Spring) by analysis of sequence variation at the small subunit RNA (SSU rRNA) and 16S-23S rRNA intervening transcribed spacer (ITS) loci. To this end we are currently exploring the two-dimensional distribution of cyanobacterial genetic variants (i.e., with Departmenth in mats at various temperature sites along the thermal gradient. This is being done in collaboration with colleagues at the University of Copenhagen Marine Biology Laboratory, who use microsensors to characterize the physical/chemical microenvironments and distribution of microbial activities (e.g. oxygenic photosynthesis) within the mats. We are also cultivating unicellular cyanobacteria (*Synechococcus spp.*) and characterizing their SSU and ITS genotypes to verify their relevance to in situ populations. Relevant isolates will be used in studies of phenotypic adaptation to temperature and other parameters that vary in the vertical aspect of the mat.

2) To evaluate the hypothesis that geographic isolation is involved in the diversification of hot spring cyanobacteria. To this end we have completed a study of the distribution of SSU rRNA and ITS genetic variants of hot spring cyanobacteria from local (e.g., within Yellowstone) to global (e.g., North America, Japan, New Zealand and Italy) scales, lineage-specific oligonucleotide hybridization probing to quantify the importance of genetically distinct types of cyanobacteria in different places,

lineage-specific polymerase chain reaction (PCR) to test distribution of putative rare genotypes, and chemical variation among springs.

3) To examine the diversity and distribution of green nonsulfur-like bacteria in mats of alkaline siliceous hot springs. To this end we have examined variation in SSU rRNA sequences at different temperatures in the Mushroom Sp. Mat, and developed and used fluorescent in situ oligonucleotide hybridization (FISH) probes to study the morphology, Departmenth distribution and physiological activities of *Chloroflexus spp.* and their as yet uncultivated relatives in this mat.

4) To examine the diversity of green sulfur-like bacteria in sulfidic hot spring microbial mats. This has been done in conjunction with Dr. Donna Bedard (RPI), who will report results separately.

5) To study the organic geochemistry of phototrophic microorganisms inhabiting sulfidic and nonsulfidic mats in Yellowstone. To this end we have collaborated with colleagues at the Netherlands Institute for Sea Research (NIOZ) to study the lipid biomarkers of *Roseiflexus castenholzii*, a filamentous phototrophic bacterium genetically related to the uncultivated type-C Chloroflexus relatives inhabiting Yellowstone alkaline siliceous mats. We have also investigated the production, consumption and stable carbon isotopic signatures of lipids and carbohydrate biomarkers in unicellular cyanobacteria and filamentous green nonsulfur-like bacteria inhabiting mats of alkaline siliceous (e.g., Octopus Sp., Mushroom Sp.) or sulfidic hot springs (e.g., several in the Mammoth Upper Terraces Group).

Findings: 1) At all sites *Synechococcus* populations were yellow-green and chlorophyll-poor at the surface, but dark green and chlorophyll-rich in deeper layers. At 55 and 60°C, different *Synechococcus* SSU rRNA-defined populations were observed at different Departmenths. At 65 and 70°C no variation in SSU rRNA-defined *Synechococcus* populations was observed. Separate clades for surface or deep-layer ITS variants suggested that *Synechococcus* populations differently adapted to light inhabit different mat layers. At 65°C both surface and subsurface clones exhibited identical ITS sequences, suggesting either a single genotype acclimated to different light conditions, or distinct genotypes that are extremely closely related

2) We have cultivated representatives of *Synechococcus* type A and B' SSU rRNA and ITS genotypes identical to those in situ. Additional ITS genotypes of these SSU rRNA genotypes, heretofore not found in situ, have also been observed, as have isolates closely related, but not identical, at the SSU rRNA locus. Type-B SSU rRNA genotypes were also recovered, but their ITS sequences are unlike those observed in situ.

3) Different predominant and diversified Synechococcus lineages occur in different countries. Type-A/B appear endemic to North America. They have undergone extensive evolutionary radiation, apparently due to both geographic and adaptive events. Type-C1 are predominant and diversified in Japan, but also rarely observed without genetic variation at low levels in a few North American springs. Type-C9 are in greatest abundance in New Zealand, where filamentous *Oscillatoria amphigranulata*-like cyanobacteria predominate. Although the filamentous cyanobacteria have diversified, unicellular cyanobacteria have not. Type-C9 *Synechococcus* were also in low abundance in North America and Japan, where their diversification is limited. Distribution patterns, combined with the lack of correlation with physical/chemical parameters, suggest that geographic isolation is important to diversification of hot spring cyanobacteria, though members of different lineages show different propensities for dispersal and colonization. The patterning of ITS sequence variation suggests that geographic isolation may be significant within Japan and the Greater Yellowstone Ecosystem.

4) We found a large diversity of *Chloroflexus* and type-C related SSU rRNA sequences. Both *Chloroflexus spp.* and type-C SSU rRNA sequences are associated with filamentous microorganisms. At 60° C, nearly all filaments were type-C organisms distributed throughout the photic zone. At 700°C, *Chloroflexus* and type-C each comprised about half of the filament population, the former restricted to the upper one mm. Type-C cells were observed to incorporate 14C-acetate, but not 14CO2 during light incubation.

5) The main lipids of *R. castenholzii* were alkane-1, 2-diol glycosides or fatty glycosides and C37-40 wax esters. *R. castenholzii*-like organisms could be the source of structurally similar compounds found in mats, though in mats glycosides are less abundant and wax esters are shorter. We are investigating the possibility that carbohydrate biosynthesis in cyanobacteria could impart a heavy isotopic signature that is transferred to GNSB. We conducted a diel study of polyhexose variation in the Mushroom Sp. mat and are in the process of determining the stable isotope signatures of polymerized sugars in each type of organism.

Project title: An Analysis of Soil Microbial Community Structure in an Evolving Thermal Soil Environment.

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Objective: The objective of this work is to use molecular methods to analyze soil microbial community succession in response to changes in soil temperature. Investigations of the biology of hydrothermal systems have added greatly to our understanding of microbial species diversity and their evolutionary relationships. However, previous studies have generally been limited to thermal systems that are well established on the time scale of human observation. The death of lodgepole pines in this study site are indicative of a very recent expansion of the underlying geothermal plumbing. In some places temperatures as high as 80°C were recorded, which only six months previously were closer to 25°C. This study site provides us with a unique opportunity to observe changes in microbial community structure as they are occur. This work will allow us to address questions concerning the forces affecting microbial community structure, diversity and the colonization of geothermal features by thermophilic microorganisms.

Findings: In this study, microbial species diversity was assessed across a landscape in Yellowstone National Park where an abrupt increase in soil temperature had occurred due to recent geothermal activity. Soil temperatures were measured and samples were taken across a temperature gradient (300–650°C) that spanned geothermally disturbed and unimpacted soils; thermally perturbed soils were visually apparent by the occurrence of dead/dying lodgepole pine trees. Changes in soil microbial diversity across the temperature gradient were qualitatively assessed based on 16S rRNA

sequence variation as detected with denaturing gradient gel electrophoresis (DGGE) using both rDNA and rRNA as PCR templates, and primers specific for Bacteria or Archaea. The impact of the major heating disturbance was apparent in that DGGE profiles from heated soils appeared less complex than the unaffected soils. Phylogenetic analysis of a bacterial 16S rDNA clone library PCR cloned from a recently heated soil showed that a majority of the clones belonged to the Acidobacterium (51%) and Planctomyces (18%) divisions. Agar plate counts of soil suspensions cultured on dilute yeast extract and R2A agar media incubated at 250°C or 500°C revealed that thermophile populations were 2–3 orders of magnitude greater in the recently heated soil. A soil microcosm laboratory experiment simulated the geothermal heating event. As determined with both RNA- and DNA-based PCR coupled with DGGE, changes in community structure (marked change in DGGE profile) of soils incubated at 500°C occurred within one week and appeared to stabilize after three weeks. The results of our molecular and culture data suggest that thermophiles or thermotolerant species are randomly distributed in this area within Yellowstone National Park and that localized thermal activity selects for them.

Project title: Viral Populations in Thermal Environments

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Objective: High temperature thermal environments (>75°C) are unusual among natural environments in their limited number of biological components and their relatively stable chemistries, temperatures and nutrient contents. The biotopes of thermal environments are comprised of the Bacterial and Archaeal cells and their cognate viruses. Few natural environments are so purely prokaryotic. It is becoming increasingly clear that viruses play a key role in the ecology of most, if not all, environments, presumably including thermal environments. While some research has been reported on thermophilic microbial populations, very little was known at the outset of this project regarding viral populations. One goal of this project is understanding the abundance and diversity of viral populations in these environments. A second goal of this work is to analyze the viruses to determine their utility for various applications.

Findings: Fifteen thermal features were examined (75–90°C, pH 4–9) for viral populations. Identity and diversity of viruses were examined by electron microscopy, environmental abundances were determined by epifluorescence microscopy and the biodiversity of their hosts was established by rDNA sequencing. For several of the thermal environments, a picture of the ecology is emerging that includes the phage diversity based on morphotypes, phage and microbial abundances based on epifluorescence microscopy, microbial diversity based on rDNA analysis. Molecular analysis of phage populations has begun.

Project title: Enhanced Practical Mitigation of Carbon Dioxide

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Additional investigator: Igor Broun

Objective: To isolate and characterize cyanobacteria from Black Sand Pools, Rabbit Creek and Mammoth Terraces that can be used in high temperature bioreactors (55°C) for the remediation of carbon dioxide from coal-fired power plants. The remediation equipment has been designed to allow its retro-fitting to the power plants. The cyanobacterial isolates have to be thermotolerant.

Findings: We have approximately 130 isolates growing at 55°C in three types of media. Eleven are unialgal. We have found that inoculum size is critical when the growth medium is gassed with a mixture containing 5% carbon dioxide and pH control is necessary. Growth curves based on chlorophyll a determinations show generation times as low as 8–10 hours and as high as 48 hours, depending on conditions (light level, carbon dioxide enrichment). The cyanobacteria were sampled from natural substrates in the aquatic environments mentioned above, or more efficiently, by allowing in situ colonization of artificial substrata. These artificial surfaces are those that are expected to be used in the pilot plant bioreactors.

Project title: Production and Consumption of Trace Gases by YNP Microbial Communities

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Objective: In order to be able to better characterize a biological signal in the composition of trace gases which may be emitted to the atmosphere, we made preliminary measurements of trace gas production by Yellowstone National Park hot spring microbial communities. It is our goal to use this information to help develop our search strategies for life through the detection of biomarkers in the atmospheres of extrasolar planets. Additionally, in preliminary work to investigate cyanobacterial sources of hydrogen, we made measurements of nitrogen fixation (using the acetylene reduction assay) by communities of the cyanobacterium *Mastigocladus laminosus*. Hydrogen production is an inevitable consequence of nitrogen fixation.

Findings: In this, our first field season, we concentrated on measurements of methane and hydrogen. The measurements of organosulfur compounds, originally planned for the 2001 field season, will instead be made in 2002.

Preliminary measurements were made on the hydrogen contents of gas bubbles emanating from the source waters of several springs, in order to gauge the potential for H2 to provide a source of chemical energy, or of reducing power, for microbes in these systems. Gas bubbles in Mammoth region springs were generally very low in H2, with concentrations consistently below 5 ppm. Highest concentrations (500–700 ppm) were observed in the acidic source waters of Norris Geyser Basin. For measurements in the Octopus Spring/White Creek and Rabbit Creek areas, a wide range of concentrations were observed (3–319 ppm). For these last areas, springs that contained significant photosynthetic microbial communities often exhibited markedly lower concentrations of H2 in the venting gas than springs that had no visible photosynthetic communities, possibly suggesting biological utilization of upwelling H2.

Methane concentration measurements were made in these same areas, both in source gas bubbles, and in bubbles of gas produced (or trapped) by microbial communities present in the springs. Methane concentrations in source water bubbles in the Mammoth area averaged around 300 ppm. Methane concentrations in the Norris Geyser Basin source gas samples averaged around 500 ppm. Higher methane concentrations were found in the Octopus Spring/White Creek and Rabbit Creek areas (averaging approximately 3,000 ppm). Methane concentrations in bubbles entrapped by microbial mats in all of the areas were much lower than concentrations of methane in source water gas bubbles. Incubations of microbial mat communities (to determine rates of methane production by these communities) confirmed that methane production rates in these mats are very low.

We used acetylene reduction assays to test for nitrogen fixation activity in two *Mastigocladus* populations, one from White Creek in the Lower Geyser Basin and the other from the Mammoth Terrace area. Water samples from White Creek lacked both ammonium and nitrate, and filaments of *Mastigocladus* contained abundant heterocysts. In contrast, the Mammoth site had high concentrations of nitrate (ca. 30 micromolar), and *Mastigocladus* had not produced heterocysts. Short-term (3 h) acetylene reduction assays demonstrated high rates of nitrogen fixation in the White Creek population (1.7 ppm ethylene produced per microgram Chlorophyll per h) and a lack of activity in the Mammoth population.

Future work: Methane could be produced biologically in the deeper, anoxic subsurface regions by organisms that utilize H2 as an energy source. Isotopic analysis of this upwelling methane, which can help to discriminate between this biological mechanism and a purely geochemical one, is proposed as future work for these sites.

Project title: Genetic Analysis of Brucella from Bison and the Generation of a PCR-based Diagnostic System for Epidemiological and Ecological Studies

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Objective: The objectives of this work are to: 1) determine the genetic complexity of *Brucella* isolates from a variety of animal hosts, 2) develop a high sensitivity PCR based diagnostic system to identify the presence of *Brucella* isolates, 3) develop a PCR based diagnostic system to track specific genotypes of the *Brucella* isolates, 4) develop a PCR based diagnostic system to discriminate live *Brucella* cells from dead cells.

In addition to the objectives listed above, studies will be performed to convert the diagnostic systems to field adaptable systems capable of simple and rapid data generation.

Findings: We have completed the genetic analysis of *Brucella* isolates from several animal hosts including bison, cattle, and elk. These data are currently being incorporated into a scientific manuscript that will be submitted in 2001. In addition, several PCR primer sets have been prepared that amplify products specifically from *Brucella abortus* isolates. Protocols have been developed for extracting *Brucella* cells from blood samples and detection using the PCR diagnostic system. The genetic analysis has indicated that the strain RB51 used for vaccine development may be genetically unstable. This raises concerns over the use of RB51 generated vaccine as this strain may have the potential for reversion to virulence. This year, a manuscript will be completed and submitted to a peer-reviewed scientific journal.

Project title: Processes Maintaining Archaeal Biodiversity in Geothermal Environments

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Objective: Analyze diversity of prokaryotes associated with acidic sulfurous hot springs.

Findings: DNA sequence analysis of a number of YNP isolates has been done by a collaborator (Rachel Whitaker, University of California, Berkeley) under the terms of a material transfer agreement. Preliminary analysis indicates some DNA sequence diversity within a species of *Sulfolobus* that correlates with geographical location within YNP and also with locations outside the Park.

Project title: Research Experience for Undergraduates: Yellowstone National Park Field Trip, Summer 2001

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Objective: The Research Experience for Undergraduates program at the Center for Biofilm Engineering, Montana State University, recruits talented students in various science, math and engineering disciplines to spend 10 weeks in Bozeman conducting biofilm research, learning effective technical communication skills and debating ethical issues that arise in technical fields of work and study. Yellowstone National Park serves as the perfect location to debate the ethics of harvesting microorganisms from natural environments. The students spent two days in the park observing wild type biofilms and discussing current biofilm research being conducted in the park.

Findings: The trip to Yellowstone Park increased the students' appreciation for field research. Viewing biofilm in a natural environment demonstrated the complex ecology associated with a living biofilm better than any bench-top laboratory system. The students left Yellowstone with a better understanding of the issues surrounding research in a national park.

Project title: Transition Between Lithouautotrophy and Chemoheterotrophy in *Sulfolobus* species

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Objective: To determine the factors, which regulate the metabolic status of hyperthermophilic archaea and bacteria in situ. To investigate methods for recovery of viable cells and the existence of postexponential phase physiological states. To assess rates of evolutionary drift in target genes.

Findings: The effect of sample pH, sample concentration, and sample ultrafiltration was examined on the recovery of viable cells from geothermal sites at various locations in the park. Preliminary evolutionary rates of drift were caculated for several target genes.

Project title: Isolation and Characterization of Thermophilic Viruses from Yellowstone National Park

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Additional investigators: George Rice, Jamie Snyder, Debbie Willits, Sue Brumfeild, Blake Wiedenheft, Kenneth Stedman

Objective: The purpose of this project is to isolate and characterize the viruses from the hyperthermophilic acidophile *Sulfolobus*. Our long term goal is to use viral analysis as a means to understand the host organism and the adaptations necessary to survive in high temperature and low pH environments. There have been no previous analyses of the viruses from *Sulfolobus* in Yellowstone. However, viruses have been previously isolated from *Sulfolobus* from Japan, Iceland, and Italy. In this study, we would like to study the diversity of *Sulfolobus* viruses from within the park, to compare viruses from Yellowstone to viruses from other parts of the world, and to find new viruses that have never been discovered before.

Findings: We are successful at culturing *Sulfolobus* from Yellowstone and isolating viruses from those cultures. We have isolated viruses that are morphologically identical to isolates from Japan, Iceland, and Italy. However, based on preliminary genetic analysis, they are different. We have also isolated viruses that seem to be completely novel. We are continuing to find viruses from Sulfolobus and analyze them for biochemical and evolutionary studies.

Project title: Ecological, Physiological, and Molecular Biological Studies of Fungi from Geothermal Soils and Thermotolerant Plants in Yellowstone National Park

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Additional investigators: Joan Henson, Regina Redman, Kathy Sheehan

Objective: The proposed research will provide information to increase our understanding of fungal survival in unique environments, the roles of fungi in ecosystem dynamics, and the temporal and spatial scales of the micro-habitats that fungi occupy. Specifically, this work will provide information about: 1) how fungi survive under environmental conditions too harsh for mycelial growth; 2) if fungal community structure changes in response to environmental conditions; 3) if fungi can alter between saprophytic and symbiotic lifestyles in response to environmental conditions; 4) the scale of soil studies necessary to accurately assess the roles of these fungi in ecosystem dynamics; 5) how biological and/or genetic diversity of fungal communities changes in response to environmen-

tal conditions; 6) the adaptive mechanisms of tolerance required for the growth of fungi soils containing high levels of metals and other inorganic chemicals.

In addition, the feasibility of developing molecular biological tools will be determined for rapidly assessing a) fungal community structure based on molecular biomass measurements; b) the metabolically active, and inactive, species of fungal communities; and c) the occurrence of fungi in thermotolerant plants.

Findings: Several fungal species have been isolated and found to be either mesophillic or thermophillic. The populations of both fluctuate throughout the year as a result of soil temperature and moisture. Fungi are in highest densities in soil under plants and can be found in soils with temperatures up to 100°C. There are two classes of fungi present in the soils: saprophytic and symbiotic. Most of the fungi are very tolerant to heavy metals. This year extensive analyses will be performed to define the molecular genetic and ecological bases of the symbioitc interactions between these fungi and plants in geothermal soils.

Project title: Diversity of Thermophiic Anaerobic Microorganisms

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Objective: Elucidation of the biogeology, ecology, phylogeny, physiology, biogeochemistry, and diversity of thermophilic microorganisms. Presently with a special focus on alkalithermophiles as a novel group of thermophiles.

Findings: We had a very successful workshop meeting in Yellowstone National Park on the sister park in Kamchatka and how to preserve the Uzon Valley and Geysir Park in Kamchatka. The workshop included the visit of several of the hot spring areas. I collected some samples from the area behind Octopus Spring for measurements of alkalinity and isolations of alkalithermophiles growing above pH (60°C) 10. However we were not able to get anything new out of those samples.

Project title: Bacteria Living at Low pH and High Temperature

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Objective: Isolation and characterization of unknown thermophilic acidophilic microbes.

Findings: *Sulfolobus*-like microbes were sampled at 880°C and pH 2.2 at a site in Crater Hills, Great Sulfur Spring.

Project title: Phylogenetic Analysis of High-Temperature Ecosystems

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Additional investigators: Jeff Walker, J. Kirk Harris, Scott Dawson, Alicia Berger, John Spear

Objective: Ongoing research continues to focus on the survey of microorganisms in Yellowstone microbial ecosystems with varying solution chemistries. We have been measuring hydrogen concentration in the bulk aqueous phase to determine the variability in H2 concentration in the Park's hotsprings. Small samples of biomass (1–10 grams) are obtained, and brought back to the lab for analysis by a molecular approach, rather than the traditional methodology of culturing. The molecular approach is based on cloning and sequencing of the small sub-unit ribosomal gene (16S rRNA gene) to determine the microbial composition of these ecosystems. Ongoing studies include analyses of sub-aqueous and sub-aerial systems for bacterial, archaeal, and eucaryal life.

Findings: Work from 1999 and 2000 on Well Y-7 in Biscuit Basin, has been written up and accepted for publication in Yellowstone Science for April 2002. We found that the sub-surface of Biscuit Basin has a varying temperature of its hot waters over the course of a year. We also found that the Well is rather devoid of life along its 250 foot length and that it has a thermal gradient of 50°C at the surface to 135°C at the bottom.

In 2001 we measured the bulk aqueous phase hydrogen concentration at a number of hot springs in the Park. We found high nM concentrations of H2 at a number of locations, indicating that hydrogen, rather than sulfur, probably drives primary productivity in this geothermal ecosystem. This is supported by the molecular microbial studies done within the Park, where the overwhelming number of organisms utilize H2 as the basis for their metabolisms. A manuscript on this work is in preparation.

Work from 1998 and 1999 on the formation of geyserite within the Park and its associated

microbiota has been completed. We see again, an extreme amount of microbial diversity, and a dependence on hydrogen. This work, with lead author Carrine Blank, has been written up and is about to be submitted to *Applied and Environmental Microbiology*.

Project title: Survey of Yellowstone Hot Springs for Green Sulfur Bacteria

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Additional investigators: David Ward, Ulrich Nuebel, Mary Bateson

Objective: 1) To survey selected hot springs in Yellowstone National Park for the presence of thermophilic green sulfur bacteria (GSB). 2) To characterize thermophilic GSB found in Yellowstone hot springs by molecular techniques. 3) To further characterize and possibly isolate organisms whose 16S ribosomal gene sequences indicate that they may be deeply branching relatives of green sulfur bacteria from selected Yellowstone hot springs.

Findings: We have continued to follow up on the GSB located in several hot springs in Yellowstone National Park in 2000. This year we revisited sites in the Mammoth Hot Springs region, Gibbon Hills area, and the Mud Volcano region. We confirmed that the GSB found last year in the latter two sites are still present although the temperatures and appearance of the sites have changed. We have not been able to demonstrate the presence of any GSB in the Mammoth Hot Springs area. We have sequenced an 800 bp region of 16S rRNA genes from GSB enrichments and from DNA extracted from hot spring mats and amplified with GSB-specific primers. We have confirmed that sequences from GSB found in two different regions, the Gibbons Hills area and the Mud Volcano region, are distinct from those of the thermophilic GSB *Chlorobium tepidum* previously identified in New Zealand. We are continuing our analyses.